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LETTER TO THE EDITOR

Plaque formation by a velogenic Newcastle disease virus in human colorectal cancer cell lines

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Newcastle disease virus (NDV) was first reported to exhibit oncolytic activities on Ehrlich's ascites carcinoma in 1955 (*1*). Since then, various studies have also shown the effectiveness of NDV as an oncolytic agent (*2*). Thus far, only the lentogenic strains of NDV, such as HUJ (*3*), Ulster (*4*), and the mesogenic strains such as 73-T (*5*), MTH-68/H (*6*) and PV701 (*7*) were evaluated in clinical studies. To the best of our knowledge, no velogenic NDV strain has been tested in clinical trials hitherto. This might be due to its velogenic properties and to the specific regulations by the World Organization for Animal Health on the use of notifiable diseases. Nonetheless, in order to allow further investigation into the pathogenesis and oncolytic properties of this NDV strain, a proper quantitative method for its infectivity is needed.

In the studies using the lentogenic and mesogenic strains, various techniques were used to measure the NDV infectivities and dosages, including the HAU (*4*) and EID_{50} (*5*), as well as the plaque assay (*7*). The plaque assay is considered as the "gold standard" for *in vitro* quantification of viral infectivity (*8*). Up to now, there are only two studies which reported the use of NDV plaque assay in human cancer cells (*7*, *9*). However, these studies used only the mesogenic NDV strains, and they did not discuss the details of the resulting plaque morphologies. In view of the need for a standard quantitative method for velogenic NDV infectivity, we screened a panel of human colorectal cancer (CRC) cell lines to determine the best cell line to be used in a plaque assay. In this study, we used a method originally used to determine the PFU titer of respiratory syncytial virus (*10*), to determine the PFU titer of a velogenic NDV strain AF2240 (*11*).

The cell lines and NDV used were examined for potential mycoplasma contamination. This was done to prevent any undesirable effects caused by the mycoplasmas. McKimm-Breschkin (*10*) reported that cells contaminated with mycoplasmas produced a 10-fold lower efficiency of viral infection and replication. In addition, the shapes of the resulting plaques also became irregular. For plaque assay, each mycoplasma-free CRC cell line, namely SW620, SW480, DLD-1, Dks8, HCT116p53$^{+/+}$, HCT116p53$^{-/-}$, and HT29 were seeded into 6-well plates (at approximately 2×10^6 cells per well) and incubated in a humidified 37°C incubator with 5% CO_2. After rinsing twice with PBS, serial 10-fold dilutions of NDV stock virus in serum-free RPMI-1640 medium (PAA Laboratories) was added into appropriate wells and incubated for one hr with rocking at 15 min intervals. Following incubation, the viral suspensions were removed and the cells were rinsed twice with PBS. Pre-warmed serum-free media was used to dilute molten molecular biology grade of agarose (Vivantis), maintained at 42°C following sterilization at 121°C for 15 min at pressure of 15 psi, to a final concentration of 0.3% (w/v). The mixture (3 ml) was then layered onto the cells and

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Abbreviations: NDV = Newcastle disease virus; CEF = chicken embryo fibroblasts; CRC = colorectal cancer

Table 1. A summary of the NDV AF2240 plaque characteristics with various CRC cell lines

Cells	Plaque			
	Diameter (mm)	Size	Visibility	PFU/ml
SW620	1.5–3.0	Uniform	Clear	3.2×10^9
SW480	1.5–4.0	Heterogeneous	Clear	3.0×10^9
Dks8	1.0	Uniform	Moderate	1.8×10^9
DLD-1	1.5-2.0	Uniform	Moderate	1.0×10^9
HCT116 $p53^{-/-}$	1.0	Uniform	Moderate	6.0×10^8
HCT116 $p53^{+/+}$	–	–	No plaque	0
HT29	–	–	No plaque	0
CEF	–	Heterogeneous	None	ND

ND = not determined.

allowed to solidify. The plate was then incubated at 37°C for 7 days. Cells were then fixed with 1% (v/v) formaldehyde in 0.15 mol/l of NaCl (2 ml) and incubated overnight to allow the fixing solution to penetrate the agarose. The agar was removed and the cells were stained with 0.05% (w/v) neutral red until plaques became visible followed by rinsing with running tap water and air-drying.

Plaques were visible in all of the cell lines tested except in HCT116$p53^{+/+}$ and HT29. The intensity of neutral red staining of HCT116 $p53^{+/+}$ cells infected with various dilutions of NDV did not show significant differences compared to that of the uninfected control. This does not necessarily indicate that the cells are resistant to NDV infection, but perhaps they are merely not showing obvious cytopathic changes. For example, another oncolytic virus, the tanapoxvirus, has been shown to infect cancer cells without displaying any CPE (*12*). Indeed, we demonstrated that the culture medium from NDV-infected HCT116 $p53^{+/+}$ cells gave visible plaques using SW620 cells (data not shown). HT29 cells did not show any plaques but displayed a significant reduction in color with the increase in NDV titer. Filman *et al.* (*13*) showed that early stages of cellular damage led to delocalization of neutral red and consequently a diffuse stain of the cytoplasm and nucleus. In the case of severe cellular damage, cells lost the ability to retain neutral red and appeared as unstained compared to that of the surrounding viable cells. Hence, in the present study, the reduced staining intensity seen in infected HT29 cultures was likely due to NDV-induced cellular damage.

Plaques formed in the other cell lines appeared to be heterogeneous in size except in Dks8 and HCT116 $p53^{-/-}$ (Table 1). In these two cell lines, the plaques were more uniform, with a size of approximately 1 mm in diameter. This plaque size, however, is too small to allow proper quantification. The plaques were also moderately opaque which caused problems in reproducibly identifying them. Plaques formed in SW620 monolayers were optimal, in that they were clear and reproducibly large. By contrast, SW480 plaques were too heterogeneous, leading to questions as to whether they represented individual plaques or the fusion of multiple plaques. Thus, SW620 represents the best choice to determine the PFU titer for NDV AF2240 in cancer cells. Plaques formed in this cell line were relatively uniform in size, ranging from 1.5 to 3.0 mm and clearly visible for counting. It is worth noting that our attempts to perform plaque assays, using SW620 cells deliberately infected with mycoplasmas resulted in complete cell death after 7 days of incubation. Hence, it was not possible to determine the PFU titre in these mycoplasma-contaminated SW620 cultures (data not shown).

NDV titration, using the plaque assay, was originally reported by Katz *et al.* (*14*). They used a monolayer of chicken embryo fibroblasts (CEF) to propagate the virus since birds are the natural host for NDV. To compare the plaques formed by NDV AF2240 in SW620 versus the CEF, we repeated the assay using a fresh stock of virus. The difference in plaque formation was dramatic. In contrast to SW620 cells, CEF gave indistinct plaques leading to great difficulty in plaque enumeration (Table 1). Therefore, we were unable to comparatively quantify the number of plaques produced in these two cell lines.

In view of the potential for velogenic NDV to be an effective oncolytic agent, we tested various human cancer cell lines to determine the most suitable candidate for viral infectivity quantification. In the present study, variable PFU titers were obtained using the different cell lines tested (Table 1). This stresses the importance of selecting the appropriate host cells for accurate quantification of NDV infectivity. These findings indicate that the numbers and morphologic characteristics of plaques depend on the cell lines used as host in the assay. Cancer cell lines, perhaps, are the most suitable cell lines to be used for the plaque assay when one is testing potential oncolytic NDV strains. However, careful selection of the cell line is crucial for successful plaque assays.

In this study, a series of CRC cell lines were used to test the plaque forming ability of a velogenic NDV strain. Among these, the SW620 cell line was found to be the optimal cell line to be used for NDV AF2240 plaque assays. These data are useful for further characterization of this virus and other velogenic NDVs which may contribute to their potential use as oncolytic viruses in clinical trials.

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References

1. Flanagan AD, Love R, Tesar W, Proc. Soc. Exp. Biol. Med. 90, 82–86, 1955.

2. Sinkovics JG, Horvath J, J. Clin. Virol. 6, 63–73, 2000.

3. Freeman AI, Zakay-Rones Z, Gomori JM, Linetsky E, Rasooly L, Greenbaum E, Rozenman-Yair S, Panet A, Libson E, Irving CS, Galun E, Siegal T, Mol. Ther. 13, 221–228, 2006. http://dx.doi.org/10.1016/j.ymthe.2005.08.016

4. Schirrmacher V, Haas C, Bonifer R, Ahlert T, Gerhards R, Ertel C, Gene Ther. 6, 63–73, 1999. http://dx.doi.org/10.1038/sj.gt.3300787

5. Cassel WA, Garrett RE, Cancer 18, 863–868, 1965. http://dx.doi.org/10.1002/1097-0142(196507)18:7<863::AID-CNCR2820180714>3.0.CO;2-V

6. Csatary LK, Moss RW, Beuth J, Töröcsik B, Szeberenyi J, Bakacs T, Anticancer Res. 19, 635–638, 1999.

7. Pecora AL, Rizvi N, Cohen GI, Meropol NJ, Sterman D, Marshall JL, Goldberg S, Gross P, O'Neil JD, Groene WS, Roberts MS, Rabin H, Bamat MK, Lorence RM, J. Clin. Oncol. 20, 2251–2266, 2002. http://dx.doi.org/10.1200/JCO.2002.08.042

8. Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, Field's Virology. 5th ed. Baltimore: Lippincott Williams & Wilkins, 2007.

9. Reichard KW, Lorence RM, Cascino CJ, Peeples ME, Walter RJ, Fernando MB, Reyes HM, Greager JA, J. Surg. Res. 52, 448–453, 1992. http://dx.doi.org/10.1016/0022-4804(92)90310-V

10. McKimm-Breschkin JL, J. Virol. Methods 120, 113–117, 2004. http://dx.doi.org/10.1016/j.jviromet.2004.02.020

11. Yusoff K, Tan WS, Avian Pathol. 30, 439–455, 2001. http://dx.doi.org/10.1080/03079450120078626

12. Lee HL, Essani K, Open Virol. J. 4, 1-6, 2010.

13. Filman DJ, Brawn RJ, Dandliker WB, J. Immunol. Methods 6, 189–207, 1975. http://dx.doi.org/10.1016/0022-1759(75)90064-2

14. Katz D, Ben-Moshe H, Alon S, J. Clin. Microbiol. 3, 227–232, 1976.